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JAPANESE PATENT OFFICE (JP)
PATENT JOURNAL (A)
KOKAI PATENT APPLICATION NO. HEI 1[1989]-226900

Int. Cl.[4]:	C 07 K 15/14 A 61 K 37/465 C 07 K 3/12 3/16 3/18 13/00 C 12 P 21/00
Sequence Nos. For Office Use:	8318-4H 8615-4C 8318-4H B-6712-4B
Filing No.:	Sho 63[1988]-54159
Filing Date:	March 8, 1988
Publication Date:	September 11, 1989
No. of Claims:	3(Total of 6 pages)
Examination request:	Not filed

METHOD FOR PURIFYING PROTEIN C

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[Amendments have been incorporated into text of translation.]

Claims

1. Method for purifying protein C, characterized by containing a process of recovery of the supernatant after isoelectric precipitation of a protein-containing solution at pH 4-6.

2. Method for purifying protein C, characterized by containing a process of elution with an organic solvent having a concentration gradient from 10 to 80 v/v% after a protein-containing solution is added to a reversed-phase chromatographic carrier at pH 1-2.

3. Method for purifying protein C, composed of the following processes: a protein-containing solution is

(a) subjected to isoelectric precipitation at pH 4-6, then the supernatant is recovered

(b) added to a cation exchanger at pH 4-6, followed by eluting with 0.1-2M salt solution

(c) added to an anion exchanger at pH 6-9, followed by eluting with a salt solution having concentration gradient of 0.2-2M

(d) gel filtration process using a carrier with a fractionation molecular weight in the range of 1000-1,000,000

(e) elution process using a salt solution having a concentration gradient of 0-2M after adding to an immobilized sulfated polysaccharide at pH 5-7

(f) elution process using an organic solvent having a concentration gradient of 10-80 v/v% after adding to a reversed-phase chromatographic carrier at a pH 1-2.

Detailed explanation of the invention

Industrial application field

The present invention pertains to a method for purifying protein C.

Prior art

Protein C is a protein containing γ-carboxyglutamic acid (Gla), which is an amino acid, synthesized in the presence of vitamin K, and it is named protein C after the fraction number "C" of the chromatography in the purification of serum. Protein C is mainly synthesized in the liver as a one-chain glycoprotein with a molecular weight of 62,000, and in circulating blood it mainly exists as a two-chain molecule (L chain and H chain). Protein C is a serine protease precursor

enzyme similar to factor IX, factor X, and factor VII of Gla-containing coagulation factor, and its structure resembles these factors, and they have approximately 40% homology.

The thrombin generated by the coagulation mechanism of living things forms a complex with thrombomodulin which is a unique receptor present in the arterial endothelium, and loses various catalysis for its original coagulation factor, and becomes entirely a strong activator of protein C. Protein C is subjected to limited degradation by the complex and becomes activated protein C (APC), which is a very efficient coagulation fibrinolysis-regulating factor having both anticoagulation and fibrinolysis stimulation. Its action mechanism is selective degradation and deactivation of F. Va and F. VIIIa, which are coenzymes in the so-called protease and prothrombinase complex on the phospholipid of the endothelial surface, to inhibit blood coagulation strongly. APC selectively degrades PAI (plasminogen activator inhibitor) of tPA inhibition factor and also promotes the fibrinolysis reaction.

For purification of protein C, several methods have been known, such as the precipitation adsorption method using barium chloride, ammonium sulfate fractionation, anion exchanger treatment, preparative electrophoresis, antibody column for protein C, antibody column for protein admixture, etc. J. Biol. Chem., Vol. 251, pp. 355-363, (1976); ibid., Vol. 258, pp. 1914-1920, (1983); J. Nara Med. Ass., Vol. 35, pp. 448-454, (1984); J. Biol. Chem., Vol. 261, pp. 11097-11105, (1986); Thromb. Haemostas., Vol. 48, pp. 1-5, (1983)).

However, the present inventors found that when the above-mentioned methods were used, either satisfactory results were not obtained in purity, yield, and working efficiency, or they were not suitable for purification of a large amount of protein.

Problems to be solved by the invention

Thus the present inventors investigated a method for efficient isolation and purification of protein C, and as a result they found that the isoelectric precipitation method and reversed-phase chromatography were effective, and furthermore, they found a method for purifying protein C using these processes, and thereby they completed the present invention.

Means to solve the problems

Namely the present inventions are as follows:

Invention 1: a method for purifying protein C, characterized by containing the process of recovering the supernatant after a protein-containing solution is subjected to isoelectric point precipitation at pH 4-6.

Invention 2: a method for purifying protein C, characterized by containing the process of elution with an organic solvent having a concentration gradient of 10-80 v/v% after a protein C-containing solution is added to a reversed-phase chromatographic carrier at pH 1-2.

Invention 3: a method for purifying protein C, characterized by containing the following processes: a protein C-containing solution is

(a) subjected to isoelectric precipitation at pH 4-6, then the supernatant is recovered,

(b) added to a cation exchanger at pH 4-6, followed by eluting with a 0.1-2M salt solution,

(c) added to an anion exchanger at pH 6-9, followed by eluting with a salt solution having a concentration gradient of 0.2-2M,

(d) subjected to a gel filtration process using a carrier with a fractionation molecular weight in the range of 1000-1,000,000,

(e) subjected to an elution process using a salt solution having a concentration gradient of 0-2M after adding to an immobilized sulfated polysaccharide at pH 5-7,

(f) subjected to an elution process using an organic solvent having a concentration gradient of 10-80 v/v% after adding to a reversed-phase chromatographic carrier at a pH 1-2.

[1] Starting materials

There is no restriction to the protein C-containing solution used in the present invention as long as it originates from human serum.

Concrete examples include fractions containing blood coagulation factor IX, fractions containing concentrated prothrombin complex, and other fractions containing protein C.

[2] Purification method

(a) Isoelectric point precipitation method

At the isoelectric point, the solubility of protein is minimal; therefore, it precipitates. By utilizing this property, the protein is purified.

Namely, in this process a protein C-containing solution is treated at pH 4-6 and then the supernatant is recovered.

At this point, the treatment temperature is 2-6°C and the treatment time is 6-24 h.

After the treatment, it is centrifuged at 5000-20,000 G for 10-60 min and then the supernatant is recovered.

(b) Treatment with cation exchangers

The protein C-containing solution obtained in the above (a) is added to a cation exchanger at pH 4-6.

For the cation exchangers, Bakerbond CB_x having a carboxymethyl group as a ligand or CM-Sepharose CL-6B can be used.

Then the protein C-containing fractions obtained by eluting with a salt solution having a concentration of 0.1-2M and at the same pH are recovered.

(c) Treatment with anion exchangers

The protein C-containing solution obtained in (b) is added to an anion exchanger at pH 6-9.

For the anion exchangers, Mono Q (QAE system) used in high–performance liquid chromatography, etc., can be used.

Then, after washing with a salt solution having a concentration of 0.2-0.3 M and the same pH, elution is carried out using a salt solution having a concentration gradient of 0.2-2M and the identical pH for recovering protein C-containing fraction.

(d) Gel filtration

The protein C-containing solution obtained in (c) is added to a gel filtration carrier having a fractionation molecular weight of 1000-1,000,000.

For such a carrier, Sapense 12 with agarose as a base material for high-performance liquid chromatography, etc., can be used.

The elution can be carried out using a solution with a pH 6-8 and salt concentration of 0.1-2M for recovering protein C-containing fraction.

(e) Treatment with immobilized sulfated polysaccharide

The protein C-containing solution obtained in (d) is added to an immobilized sulfated polysaccharide under the condition of pH 5-7.

Dextran sulfate, Sepharose CL4B with heparin as a ligand, etc., may be used for the immobilized sulfated polysaccharide.

Then elution can be carried out using a salt solution having a concentration gradient of 0-2.0M and identical pH for recovering protein C-containing fraction.

(f) Reversed-phase chromatography

The protein C-containing solution obtained in (e) is added to a reversed-phase chromatographic carrier under the condition of pH 1-2.

For the reversed-phase chromatographic carrier, a reversed-phase column using $C_{[illegible]}$ is a ligand can be used.

Then elution can be carried out using an organic solvent (such as acetonitrile, methanol, ethanol, propanol, dioxane, etc.) with concentration gradient from 10-80 v/v% and identical pH for recovering protein C-containing fraction.

After each process is carried out, if necessary, ammonium sulfate fractionation or dialysis can be carried out.

Namely, the ammonium sulfate fractionation can be carried out by treating the protein C-containing solution with a 60-80% ammonium sulfate saturated solution, and then the precipitate fraction is recovered.

The dialysis can be carried out under the conditions of pH 4-7, 2-6°C, and 6-24 hours.

In the present invention, furthermore, it is possible to combine it with known purification processes.

The thus-obtained protein C can be used as a biochemical reagent and pharmacological reagent, and if it is to be used as a pharmaceutical, or in sterilization, bacteria removal, or freeze-drying, it can be carried out using known techniques used in preparing pharmaceuticals, and then the pharmaceutical can be prepared.

Effect

According to the present invention, protein C can be efficiently isolated and purified. Moreover, the operation of the method of the present invention is simple and it can be used in large-scale preparation. Accordingly, it is very useful as a method for preparing protein C.

Application examples

In order to explain the present invention in more detail, application examples will be used; however, the present invention is not limited to these application examples.

[1] Purification method

(1) 10 mL of distilled water were added to one vial of Chrismycin [transliteration] formulation and after complete dissolution, dialysis was carried out using a 20 mM sodium acetate buffer solution (pH was 5.0). After the dialysis it was centrifuged at 10,000 G for 20 min and then the supernatant was recovered.

(2) Cation-exchange chromatography

The supernatant fraction was added to a Bakerbond CB_x. For the starting buffer solution, 20 mM sodium acetate solution (pH 5.0) was used, and for the eluting buffer solution 1M sodium chloride-20 mM sodium acetate solution (pH 5.0) was used. After the addition of a sample, it was washed with the starting buffer solution and then it was switched to the eluting buffer solution for recovering the passed fraction.

(3) Anion-exchange chromatography

The dialyzate (solution obtained from dialysis) was added to a Mono Q HR 60/10 (or Q Sepharose High Performance 60/100). A 20 mM tris-HCL buffer solution (pH 7.15) was used for the starting buffer solution, and 1M sodium chloride-containing 20 mM tris-HCL buffer solution (pH 7.15) was used for the eluting buffer solution. After the sample was added, it was washed with 30% (v/v) eluting buffer/starting buffer and then linear gradient elution was carried out using 30% → 40% eluting buffer solution/starting buffer solution for recovering the active fraction.

(4) Gel-filtration chromatography

The dialyzate obtained was added to Superose 12 prep grade 60/600; then, 20 mM tris-HCL buffer solution containing 500 mM sodium chloride (pH 7.2) was used as an eluting buffer solution and the active fraction was recovered.

(5) Dextran sulfate chromatography

The dialyzate was added to Sepharose CL4B with dextran sulfate as a ligand. 25 mM imidazole (pH 6) was used as a starting buffer solution, and 1M sodium chloride-containing 25 mM imidazole (pH 6.0) was used as an eluting buffer solution. After the addition of the sample, a linear gradient elution with 0% → 50% eluting buffer solution/starting buffer solution was carried out and the active fraction was recovered.

(6) Reversed-phase chromatography

The thus-obtained fraction was added to a reversed-phase column with C8 as a ligand. Stock solutions A (distilled water containing 0.1% tetrafluoroacetic acid) and B (prepared by diluting 800 mL acetonitrile with solution A to 1,000 mL) were prepared. Solution A was added to 400 mL of solution B to a total volume of 1000 mL (final concentration of acetonitrile was 32%) and then the thus-obtained mixture was used as a starting buffer solution, and solution A was added to 500 mL of solution B to a total volume of 1000 mL (final concentration of acetonitrile was 40%) and then the thus-obtained mixture was used as an eluting buffer solution. After washing with the starting buffer solution, a linear gradient elution with 0% → 50% eluting buffer solution (the actual acetonitrile concentration was 32% → 36%) was carried out. After the fractionation, the organic solvent in each fraction was dried under reduced pressure.

[2] Measurements

(1) Ouchterlony's method

10 μL each of the sample and various antisera were added to 1.2% agar gel which was prepared using a general method. After it was allowed to stand at 4°C overnight and after confirming the formation of a sedimentation line, said gel was dehydrated with filter paper and washed with physiological saline solution; then, the dehydration and washing were repeated twice, and Coomassie staining was carried out using a common method.

(2) Immunoelectrophoretic method

1.2% agar gel was prepared (pH 8.6, $\mu = 0.06$) and after 10 μL of a sample were added, electrophoresis was carried out at 4°C for 4 h at 2 mA per 1 cm gel width. After the electrophoresis, 100 μL antiserum were added to each channel and then allowed to stand at 4°C overnight, and after confirming the formation of a sedimentation line, said gel was dehydrated with a filter paper and washed with a physiological saline solution; then, the dehydration and washing were repeated twice, and Coomassie staining was carried out using a common method.

(3) Method for measuring activity

A sample was diluted with 20 mM tris-HCL buffer solution containing 100 mM sodium chloride (pH 8.5), then it was used for measurement. Protac C (3 units/vial) was diluted with 12 mL distilled water and used. The coloring substrate MCA-3112 v was used after the contents of a vial (5.1 mg, 7.0 μmol) were dissolved completely in 1 mL dimethyl sulfoxide, then it was diluted with 85 mL 20 mM tris-HCL buffer solution (pH 8.5). A 20% (v/v) aqueous acetic acid solution was used for terminating the reaction. The flow chart for the measurement is shown in Table 1.

Table 1. Method for analyzing protein C using Protac C

//insert, p. 5//

Key:
1 Protein C sample
2 Protac C
3 Incubation at 30°C for 20 min
4 Acetic acid
5 Measurement

The purification steps, activity recovery, and degree of purification are shown in Table 2.

Table 2

//insert Table II, p.5//

Key:
1 Step
2 Volume
3 PC activity
4 Yield
5 Degree of purification (blood plasma = 1)
6 Concentrated solution of prothrombin complex
7 Isoelectric precipitation
8 Cation-exchange chromatography
9 Anion-exchange chromatography
10 Gel-filtration chromatography
11 Dextran sulfate chromatography
12 Reversed-phase chromatography

[4] [sic; 3] Physical and chemical properties of purified protein C

The results of molecular weight measurement using the SDS-PAGE method show that under nonreductive SDS-PAGE, a band was recognized at molecular weight 62,000, and under reduction conditions, two bands comprising an H band at molecular weight 41,000 and an L band at molecular weight 21,000 were recognized.

In the immunoelectrophoresis, one clear sedimentation line was recognized only at the α-position, and no sedimentation line was formed for antihuman PS, F. II, F. IX, F. X rabbit serum including antinormal human serum and rabbit serum.

The results of isoelectric focusing (isoelectric point electrophoresis) showed that protein C was recognized as a continuous band between pI 3.8 and 4.2.

From the above results, we presumed the purity of the isolated protein C to be 99% or greater.